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Microalgae treatment removes nutrients and reduces ecotoxicity of diluted piggery digestate

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Abstract

Liquid digestate is considered as an important by-product of anaerobic digestion of agriculture wastes. Currently, it is very often directly spread on local agricultural land. Yet recently concerns on its environmental risk of this processing has begun to rise. On the other hand, investigations on the effectiveness of microalgae for wastewater treatment have started to consider also this complex matrix. In this study, we cultured the green alga *Chlorella vulgaris* in diluted digestate coming from the anaerobic digestion of pig slurry and corn, with the aim to significantly reduce its toxicity and its very high nutrient concentration. For this purpose, a battery of toxicity tests composed of four acute and two chronic bioassays was applied after the alga cultivation. Results were compared with those obtained in the initial characterization of the digestate. Results show that highly diluted piggery digestate can be a suitable medium for culturing microalgae, as we obtained a high removal efficiency (> 90%) for ammonia, total nitrogen and phosphate, though after a few days phosphorus limitation occurred. Toxicity was significantly reduced for all the organisms tested. Possible solutions for optimizing this approach avoiding high dilution rates are discussed.

KEY WORDS: *Chlorella vulgaris, ammonia toxicity, algal productivity, removal efficiency, fertilizers, ecotoxicology*

1.Introduction

The interest in microalgae application for a variety of use has dramatically increased over the last decade, due to their high photosynthetic efficiency in CO_2 fixation, growth rates and biomass production (Šoštaric et al., 2009). In the environmental field, coupling microalgae growth with wastewater treatment appears to be a promising solution to overcome current high costs of microalgae cultivation and, at the same time, to solve specific treatment problem such as the compliance to National standards of nutrient concentration in effluents. With this regard, agriculture wastes appear to be good candidates and, among others, the digestate obtained through the anaerobic digestion process is particularly promising for its high content of mineralized nutrients (Li et al., 2016). Anaerobic digesters have spread in the past decade in the European countries as they have been promoted as a way to produce biogas and reduce reliance on synthetic fertilisers. In Italy, for instance, the number of farm- scale anaerobic digesters has increased from less than 50 to more than 300 in 10 years (Piccinini and Vismara, 2011).

Several studies have tested algal strains for the treatment of digestate (Xia and Murphy, 2016). Among others, the green alga *Chlorella vulgaris* has shown a good potential for nutrient removal from various types of digestate: dairy manure (Wang et al. 2010); cattle slurry and raw cheese whey (Franchino et al., 2013); municipal WWTP (Cho et al., 2013). These studies report removal rates between 63 and 94% for phosphates, and nearly 100% for ammonia.

However, particularly high ammonia concentration like that found in pig manure remains a major challenge because it is often responsible for microalgal growth inhibition (Tigini et al., 2016). Moreover, it can pose a serious environmental issue as in Europe pig farming is a major agricultural industry and many large centralised pig farms have been established. As a result, a large amount of pig manure containing high concentrations of nutrients and solids

is produced annually. As pointed out by Nkoa (2014), digestate can be toxic for the environment by several causal compounds: ammonia, volatile organic loads, salts and heavy metals. Moreover, an excessive land application of fertilisers can cause a diffuse groundwater contamination (Capri et al., 2009). A few studies included ecotoxicological assessment of agro-zootechnical digestate, mostly focused on phytotoxicity test (Gell et al., 2011; Di Maria et al., 2014) but none of them applied a battery of bioassays to check the toxicity before and after a treatment with microalgae. The ecotoxicological approach is the most suitable one to indirectly detect toxic substances and assess the ecological risk related to the land application of digestate. Moreover, it can be a key element to assess the effectiveness of innovative and environmentally sustainable wastewater treatment such as that with microalgae and the need for additional biological-based treatments. To our knowledge, this is the first study to combine phycoremediation and ecotoxicology to evaluate the environmental compatibility of agro-zootechnical wastes.

The purpose of this study is to test whether the green alga *C. vulgaris* can significantly enhance digestate's suitability for its release into the environment, in terms of nutrient removal and ecotoxicity reduction. Indeed the same piggery digestate recently analyzed through a battery of bioassays (Tigini et al., 2016) resulted as highly toxic. A secondary aim is to find a cheap and easily available substrate for microalgae cultivation in order to exploit their biomass.

2. Material and methods

2.1 Microalgae growth and nutrient removal in diluted digestate

Digestate was obtained from the effluent of an anaerobic digester, which treats pig slurry and corn, located in North West Italy. It has recently been characterized from a chemical

and ecotoxicological point of view (Tigini et al., 2016). The chemical characterization is reported in table 1.

Table 1: Digestate chemical characterization (from Tigini et al., 2016)

pH	8.0
Conductivity (mS cm^{-1})	26.7
Nitrate NO_3^--N (mg L^{-1})	229.5
Ammonia NH_4^+-N (mg L^{-1})	2050
Total Nitrogen TN (mg L^{-1})	3355
Phosphate PO_4^{3-}-P (mg L^{-1})	318.5
COD (mg L^{-1})	17600

Chlorella vulgaris CCAP 211/11b (Culture Collection of Algae and Protozoa, Argyll, UK) was selected for this study in view of our previous experiences with digestates (Franchino et al., 2013). Firstly, *C. vulgaris* was pre-grown in 250 mL Erlenmeyer flasks containing 100 mL of a modified BG11 medium (MBG11, Bona et al., 2014). Inocula for the experiments were obtained by centrifugation (2000 rpm for 30 min) of biomass grown in flasks and subsequently resuspended in 800 mL glass bubble tubes containing 500 mL of MBG11. A continuous flow of air:CO_2 (97:3 v/v) was provided in order to control pH, ensure CO_2 sufficiency and mix the culture. A temperature of 25 ± 2 °C and an average continuous light intensity of 300 µmol m^{-2} s^{-1} were maintained.

In view of its high toxicity (Tigini et al., 2016), digestate was diluted with tap water at four different concentrations: 5% (5% digestate, 95% water, hereafter 5DIG), 10% (10DIG), 20% (20DIG) and 40% (40DIG). MBG11 was chosen as control. The initial microalgae biomass concentration was 0.15 g L^{-1} (dry weight). We performed preliminary experiments of *Chlorella* growth with 4 replicates in 250 ml flasks (data not reported) which showed a good repeatability (standard deviation 0.1 and 0.07 for 5DIG and 10DIG, respectively). After this preliminary phase, tests were performed in the bubble tubes with the same temperature, light intensity and air: CO_2 described above. pH and conductivity were periodically measured with a WTW Multi340i. The test duration depended on the growth and the nutrient removal trend. We performed all experiments in duplicate and reported average values in the results.

Culture growth was estimated by measuring the dry weight (DW). For this purpose, three samples were taken from each bubble tube three times a week for gravimetric determination of the biomass concentration according to Chini Zitelli et al. (2000). Daily biomass productivity was calculated dividing the difference between the DW of two sample points by the time elapsed between the selected points.

To evaluate the nutrient removal during the experiments a portion of the samples collected for the biomass was centrifuged at 3500 rpm for 35 min and the supernatants were used to measure ammonium, nitrate, total nitrogen, phosphate and COD concentration following APAT-IRSA CNR standard methods (2003) for nutrients and ISPRA (2014) for COD. Removal efficiency (RE) was estimated by dividing the difference between the initial nutrient concentration and that on day n (C_i- C_n) by C_i, and then multiplied by 100, while the Elimination Capacity (EC) by dividing the difference between the nutrient concentration of two subsequent samples (C_{n-1} and C_n) by the time elapsed between them.

The whole experimental design is reported in figure 1.

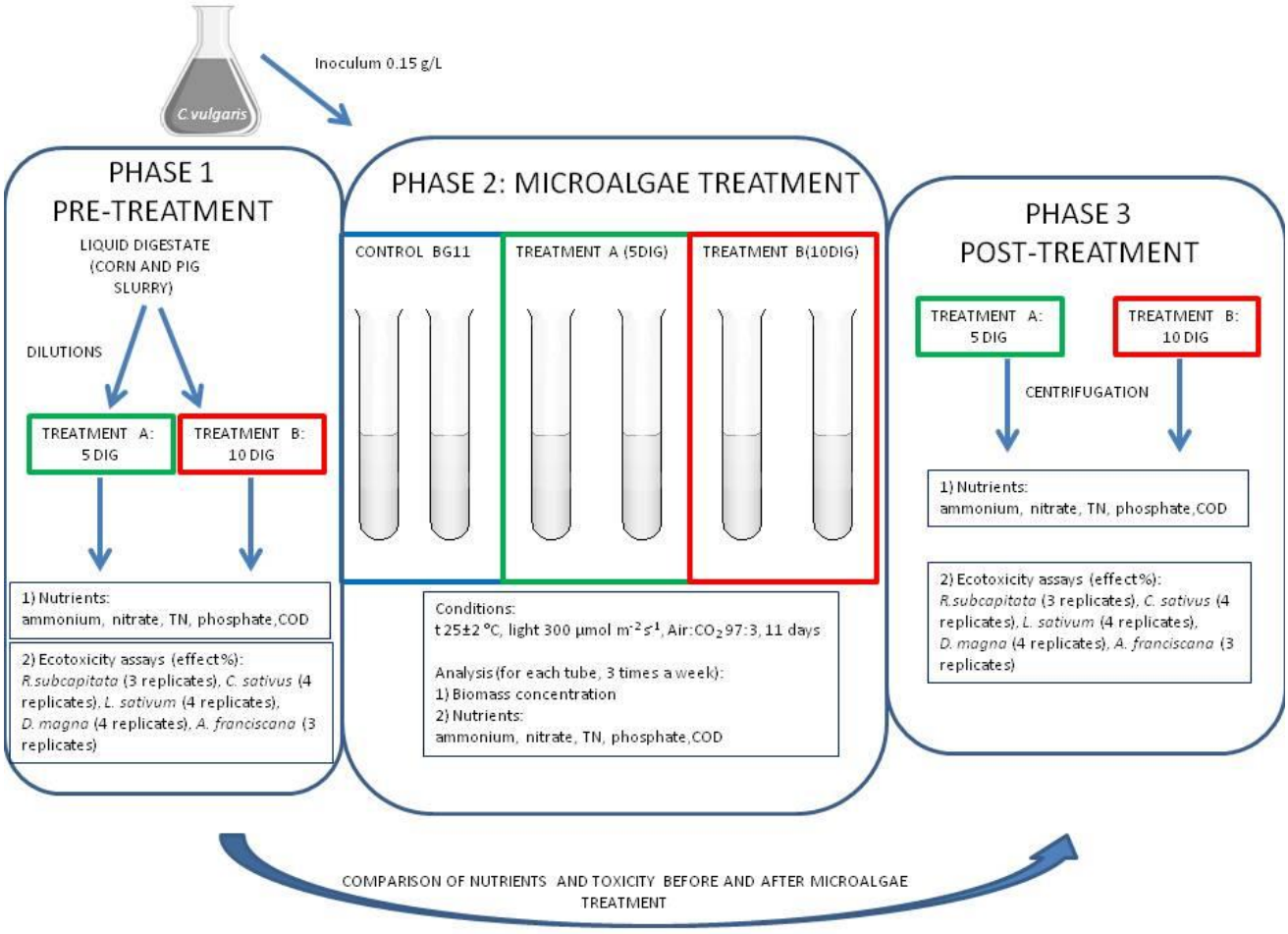


Fig.1 The experimental set-up for evaluating the diluted digestate (5% and 10%) treatment with *Chlorella vulgaris*.

2.2 Ecotoxicological evaluation before and after algal treatment

At the beginning of the experiments and at the end of the *C. vulgaris* treatment, a battery of tests was performed to assess the (residual) toxicity in 5DIG and 10DIG after the algal treatment. We selected the same tests as in the pre-treatment phase, composing a battery of four acute and two chronic ecotoxicity assays. These tests were: *Raphidocelis subcapitata* (chronic alga test); *Cucumis sativus* and *Lepidium sativum* (plant acute tests);

Daphnia magna (acute 24h and chronic 48h tests) and *Artemia franciscana* (acute test). All testing protocols have already been described in Tigini et al. (2016).

We expressed the results in terms of effect % and compared them to those obtained before the algal treatment.

2.3 Statistical analysis

Generalized Linear Models (GLMs) were used to test the effects of different substrate dilutions at different days on the biomass growth of *C. vulgaris*. GLMs were carried out with R 3.2.0 (R Development Core Team, 2015).The variables included in our GLMs were: (1) the biomass concentration, the response variable; (2) the concentration, a categorical predictor variable with 3 levels (control treatement, 5DIG and 10DIG) and (3) time, a second predictor variable. Each model thus estimates four parameters: the Y-intercept; two regression coefficients for each level of the categorical variable dilution rate, compared with one level taken as reference level; the regression coefficient for the time predictor variable. In general, the regression coefficients (β) estimated by these models represent the difference in the predicted value of the response variable for each one-unit difference in the predictor variable (e.g. the rate of change of biomass concentration with increasing time). For categorical variables such as the digestate concentration, the regression coefficients are the average difference in biomass concentration between the reference level (e.g. the control treatement) and each comparison level.

For each β value, the ratio between the estimate and its standard error is used as Wald statistic to finally assess the statistical significance.

3. Results and discussion

3.1 Microalgae growth

Microalgae growth was tested by using four different digestate concentrations as culture media: 5DIG, 10DIG, 20DIG and 40DIG. At 20% and 40% concentrations, microalgae did not grow (data not shown). In figure 2 we report results of biomass concentration during experiments with 5DIG and 10DIG.

The test lasted 11 days. Results of GLM applied to algal biomass (Annex I) confirmed that the algal concentration in control (MBG11 medium) and digestate dilutions are significantly different ($p<0.001$) considering time as an explanatory variable; moreover differences are significant also between 5DIG and 10DIG ($p<0.01$).

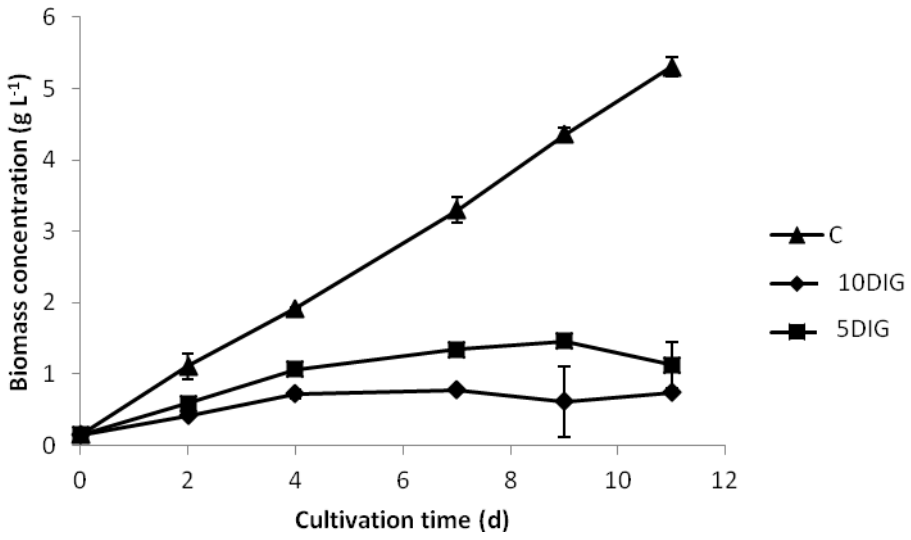


Fig. 2. *Chlorella vulgaris* growth curves in control (C), 5% (5DIG) and 10% digestate (10DIG). Error bars are standard deviations.

In detail, in the first four days the daily biomass productivities of 5DIG and 10DIG were 229 and 143 mg $L^{-1}d^{-1}$, respectively; therefore microalgae in 5DIG grew 38% more than in 10DIG. In comparison to the control (443 mg $L^{-1}d^{-1}$) the digestate reduced the growth by

68% (10DIG) and 48% (5DIG). After the fourth day, the stationary phase occurred for 10DIG, while 5DIG grew until day 9, but with a lower daily productivity (77 mg $L^{-1}d^{-1}$). The 5DIG and 10DIG highest biomass concentrations were 1.47 ± 0.08 and 0.78 ± 0.03 g L^{-1}, respectively; therefore the increase of digestate concentration limited microalgae growth of 47%, confirming its high toxicity.

Despite the lower biomass obtained compared to the control, the growth in digestate can be considered as relatively high if compared to the few previous studies carried out with pig digestate. Indeed Park et al. (2010) found lower biomass productivities (45.8-55.6 mg $L^{-1}d^{-1}$) cultivating *Scenedesmus obliquus* in diluted piggery digestate containing a lower ammonia concentration (120 ppm NH_4-N). While, the specific growth rate of *C. vulgaris* cultivated in diluted piggery digestate by Kumar et al. (2010) was comparable to that found in our study, but using a higher dilution (2% digestate) The comparison with microalgae growth data of other studies is not straightforward, due to the wide range of cultivation conditions applied (namely cultivation system, working volume, light path and intensity, temperature) that can affect biomass production. Nevertheless, the comparison with digestates coming from other matrices can offer the opportunity to shed some light on the suitability of this matrix as a culture medium for microalgae. In comparison with the previous study carried out in our laboratory with cattle digestate (Franchino et al., 2013), which contained approximately 30% less ammonia than our piggery digestate, we obtained a lower biomass productivity. Singh et al. (2011) tested microalgae growth in 4, 6 and 8% diluted anaerobically digested poultry litter effluent. The highest biomass concentration was reached in 6% dilution, because nutrients concentration in 4% was insufficient and the darker colour of 8% limited light penetration in the culture. This study confirmed the need for a high digestate dilution. Results obtained with *S. obliquus* in the liquid phase of anaerobic digester effluent from a wastewater treatment plant (Uggetti et al., 2014) showed that the increase of the ammonium

concentration up to 260 mg L^{-1} was responsible for a 77% reduction of growth rate, which was similar to the inhibition found in our study.

We can conclude that the best growth of C. vulgaris was obtained in 5DIG , but that this strain can be cultivated in medium containing up to 10% of digestate.

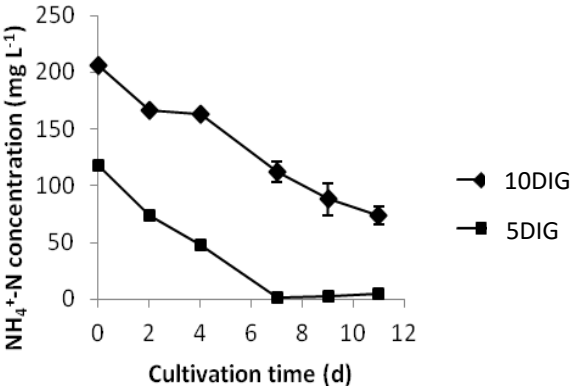
Lower dilutions make this medium unsuitable for a sufficient algal growth, for a series of possible reasons: unsuitable nutrient concentration, colour, toxicity due to ammonia or other toxic compounds.

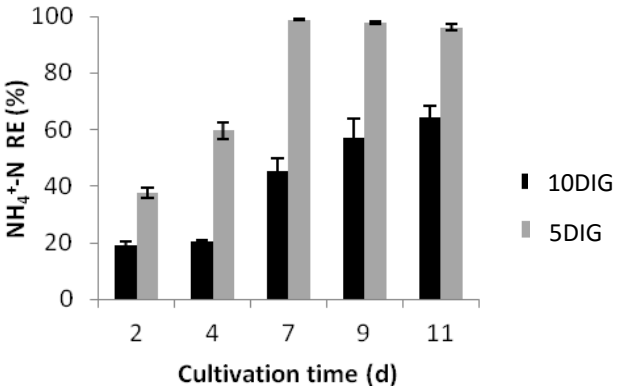
3.2 Microalgae nutrients removal

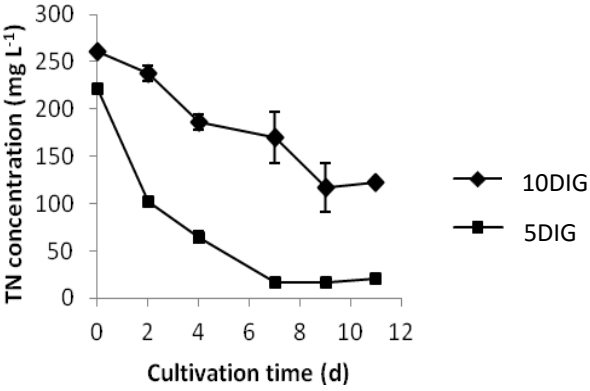
In 20DIG and 40DIG, microalgae did not grow and nutrients concentration remained almost constant for the whole experiment (data not shown). After microalgae treatment, conductivity halved in both dilutions, from 2.67 to 1.86 and from 1.34 to 0.64 mS cm^{-1} in 10DIG and 5DIG, respectively showing a strong decrease in soluble ions.

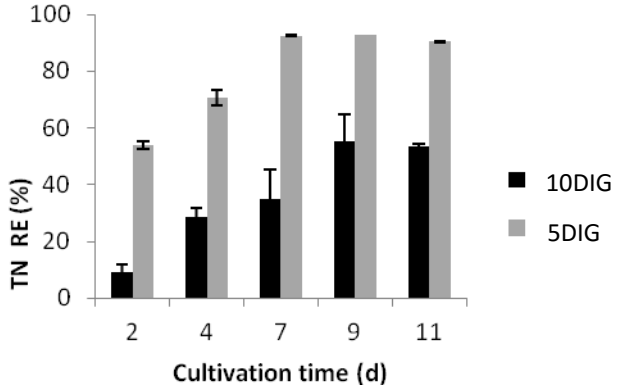
Temporal profiles of ammonium, TN, phosphate and COD removal in 5DIG and 10DIG are shown in figure 3.

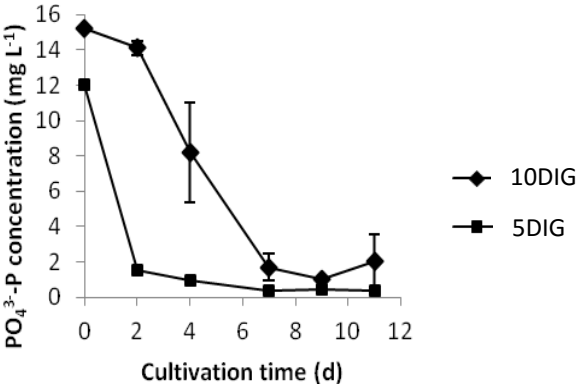
a) b)

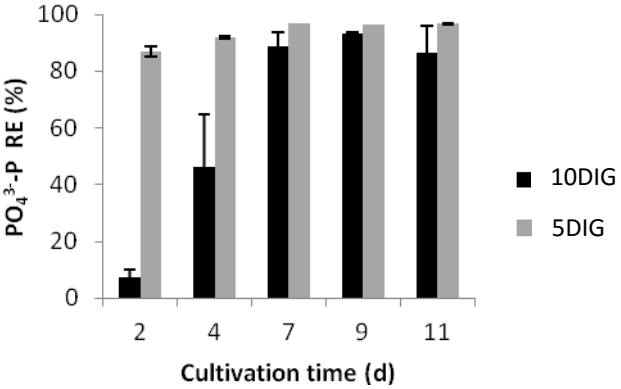

NH_4^+-N concentration (mg L^{-1})
Cultivation time (d)
10DIG
5DIG

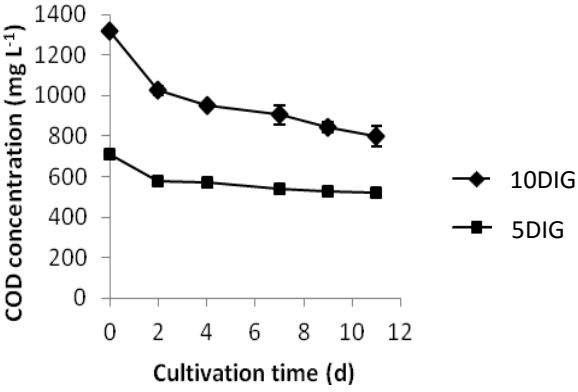

NH_4^+-N RE (%)
Cultivation time (d)
10DIG
5DIG


TN concentration (mg L^{-1})
Cultivation time (d)
10DIG
5DIG


TN RE (%)
Cultivation time (d)
10DIG
5DIG


PO_4^{3-}-P concentration (mg L^{-1})
Cultivation time (d)
10DIG
5DIG


PO_4^{3-}-P RE (%)
Cultivation time (d)
10DIG
5DIG


COD concentration (mg L^{-1})
Cultivation time (d)
10DIG
5DIG

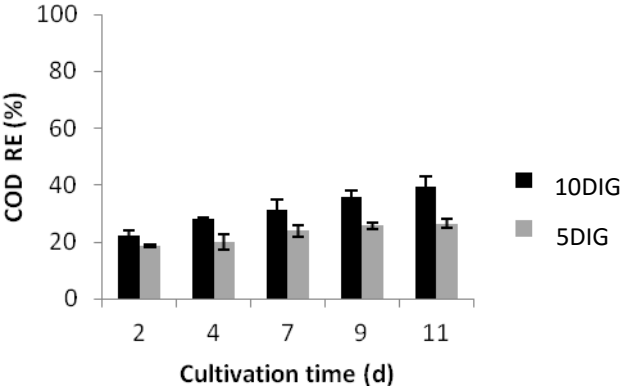

COD RE (%)
Cultivation time (d)
10DIG
5DIG

Fig. 3. Nutrients concentration (a) and removal efficiency (RE) (b) of *Chlorella vulgaris* in 5% (5DIG) and 10% digestate (10DIG). Error bars are standard deviations. Ammonia, total nitrogen and phosphates (PO_3^{4-}-P) started from very high values and showed a constant decrease. In 5DIG, the removal efficiency reached values near 100% after 7 days. COD removal was generally low at both concentrations with a slight higher efficiency at 10DIG.

Most of the nitrogen in the digestate was in the form of ammonia, as found also in other studies (Cai et al., 2013; Franchino et al., 2013; Uggetti et al., 2014). Nitrate represents only 4.8% and 2.7% of TN in 10DIG and 5DIG, respectively. Nitrate concentration remained almost constant for the whole experiment (data not shown), while ammonia progressively dropped to less than 10% in 5DIG and 40% in 10DIG. Indeed ammonia is the preferred source of nitrogen for microalgae metabolism (Cai et al., 2013), and it is by far the most abundant form in the digestate. TN concentration followed the trend of ammonia.

The highest REs (> 90%) were reached in 5DIG for ammonium, TN and phosphate, between the day 7 and the day 9 (Fig. 3). Phosphate was removed much faster and its RE after two days was already 87.1%. This means that from the 3rd day onwards phosphate starvation occurred; thus we can assert that phosphate was the limiting factor for the algal growth in 5DIG. Despite this limitation, microalgae were able to grow and remove

ammonium until day 9, when also ammonium was almost completely used. Only 26.5% of COD was removed.

REs of 10DIG were lower than that obtained in 5DIG: 64.3% of ammonium, 55.2% of TN and 93.3% of phosphate. Phosphate was the limiting factor also here, as it was consumed in the first 7 days, after that the stationary phase of microalgae growth occurred. In this treatment, microalgae maintained the stationary phase under phosphate starvation, continuing to consume nitrogen, but they were not able to significantly increase the biomass concentration as in 5DIG. From the temporal trend of nutrient removal, we observe that in 5DIG most nutrients are almost completely removed within 7 days. At lower dilution, the time span of 11 days seems to be adequate even if a complete removal was not achieved.

In addition, nutrients Elimination Capacities (EC) were higher in 5DIG, in terms of both mean and maximum value (Table 2). This is probably due to the lower toxicity of the substrate that allows a higher microalgae growth and a more rapid nutrient removal. Usually, highest values are found within the first days of the experiment, when nutrients concentration is higher. The comparison with previous studies highlights a good performance of *C. vulgaris* also in terms of nutrient removal. Park et al. (2010) found a lower EC with *S. obliquus,* grown in diluted piggery digestate (5.20 - 6.46 mg L^{-1} d^{-1}). Kumar et al. (2010) set 20 mg L^{-1} as the proper ammonium nitrogen level for optimum algal growth, as they found that higher or lower concentrations negatively affected biomass production. In our experiments, despite a much higher ammonium concentration, similar specific growth rates were obtained, while EC was more than 7 times higher. In our previous study performed with cattle digestate (Franchino et al., 2013), higher ERs were reached in 10% digestate (ammonium and phosphates removal higher than 99 and 97%), confirming the higher toxicity of our piggery digestate.

Table 2

Nutrients Elimination Capacities (EC) of microalgae grown in 5% (5DIG) and 10% (10DIG) digestates.

	EC (mgL^1d^{-1})	Nitrate $NO_3^{--}N$	Ammonia NH_4^+-N	Total Nitrogen TN	Phosphate PO_4^{3-}-P	COD
5DIG	mean	0	15.9	33.7	2.4	32.9
	max	0.1	22.3	59.5	5.2	66.8
10DIG	mean	0.2	13.7	14.4	1.4	79.8
	max	0.4	19.5	18.8	1.9	147.5

We compared our results to the Italian threshold limit values for wastewater (DLgs 152/06). Data obtained in 5DIG were in accordance with the limit values imposed for discharges in surface water, for all the parameters analyzed with the exception of COD concentration (523.5 mg L^{-1}) that was higher than both surface (160 mg L^{-1}) and sewer limits (500 mg L^{-1}). In 10DIG, only nitrate nitrogen and phosphate were below the threshold limit values, while ammonia (73.6 mg L^{-1}) and COD concentration (801 mg L^{-1}) exceeded limits for the discharged in both surface water (15 and 160 mg L^{-1}, respectively) and sewer (30 and 500 mg L^{-1}).

Results show that microalgae can be an effective method to treat 5DIG, while in 10DIG the nutrients removal was limited, probably due to the higher toxicity of the substrate and to the phosphate starvation. COD remains a critical point for both treatments.

3.3 *Ecotoxicological evaluation after C. vulgaris treatment*

In addition to nutrients removal, we assessed the toxicity reduction after microalgae treatment. Table 3 reports the results of the ecotoxicological battery applied to 10DIG and

5DIG before and after microalgae treatment. After microalgae treatment, the two least sensitive tests (*C. sativus* and *A. franciscana)* confirm the absence of significant toxic effects, with a stimulation effect for *C. sativus* in 5DIG. The response of *R. subcapitata, L. sativum* and *D. magna* indicated a substantial toxicity reduction, especially in 5DIG. Indeed the most sensitive organism *R. subcapitata* showed a toxicity reduction of 73.6% (in 10DIG) and 81.7% (in 5DIG), while using *D. magna* a toxicity reduction of 35% was found in 10DIG after 24h, and a complete disappearence of toxicity was observed in 5DIG. Using *L. sativum*, the 10DIG final toxicity was approximately one third of the initial one, and in 5DIG it was almost as low as 10% of the initial one.

Results show that the nutrients removal is associated with a toxicity reduction; therefore the high initial toxicity was probably due to the high conductivity and ammonia concentration. These findings highlight the effectiveness of microalgae application for digestate treatment in terms of both nutrients removal and toxicity reduction, thus potentially reducing the multiple negative impacts of ammonia deposition linked to digestate land application, such as surface water eutrophication, soil acidification and phytotoxicity (Nkoa, 2014).

Table 3

Ecotoxicological characterization of 10% (10DIG) and 5% (5DIG) digestates before and after microalgae treatment. Data are expressed as effect %.

	10DIG before	10DIG after	5DIG before	5DIG after
R. subcapitata growth	100	26.4	100	18.3
L. sativum root development	96.0	27.7	74.0	8.6
C. sativus root development	7.0	9.0	-15.0*	-19.3*
D. magna immobilisation 24 h	100	65	100	0
D. magna immobilisation 48 h	100	100	100	0
A. franciscana immobilisation	3.3	3.3	0	0

*biostimulation effect

4. Conclusions

Our study demonstrates that diluted piggery digestate can be a suitable culture medium for freshwater microalgae. The algal treatment had beneficial effects in reducing both nutrient concentration and toxicity to plant and animal organisms. The best results were obtained with 5DIG where after the algal growth the RE resulted higher than 90% for phosphate and nitrogen and the toxicity was almost nullified. We can argue that digestate, at appropriate dilutions, can be a cheap and efficient substitute of artificial cultivation media. This can also represent an opportunity for agricultural digestate valorization, in a perspective of circular economy.

Moreover the algal growth resulted higher than other studies on piggery digestate, thus *Chlorella vulgaris* can be considered as a suitable species for this substrate. Engineering of high- performance strains could be a further approach to increase the cell nutrient uptake. After the algal treatment, we obtained a significant reduction of toxicity, ranging from 73.6 to 81.7% for the organisms that had showed the highest sensitivity to untreated digestate

Future researches should consider the following main issues, which are closely linked: 1) the ratio N/P in our samples proved to be too high leading to P limitation after a few days of algal cultivation. This happened also in other studies, as reported in the review of Xia and Murphy (2016). A possible solution can be the dilution of digestate with wastewater containing a very low N/P ratio and turbidity; 2) the use of wastewater reduces the consume of freshwater to dilute the digestate thus making the process more attractive for industrial application; 3) dilution is so far the most applied strategy to reduce the inhibition effect mainly due to high turbidity and ammonia concentration (Xia and Murphy, 2016). We adopted this straightforward strategy because of the paucity of studies on piggery digestate and its feasibility in view of performing toxicity assessment. In light of our results, we strongly suggest a future research strategy that includes a biological–based pre-treatment

of digestate (e.g. with heterotrophic organisms) before the microalgal cultivation. With that approach, it will be possible to drop COD concentration and improve the digestate suitability for algal growth in terms of turbidity reduction and micropollutants, thus avoiding strong dilutions of the samples.

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References

1. APAT- IRSA CNR. Metodi analitici per le acque, vol I. Rapporti 29/2003. Roma: APAT; 2003.
2. Bona F, Capuzzo A, Franchino M, Maffei ME. Semicontinuous nitrogen limitation as convenient operation strategy to maximize fatty acid production in *Neochloris oleoabundans*. Algal Res, 2014; 5:1-6.
3. Cai T, Park SY, Li Y. Nutrient recovery from wastewater streams by microalgae: status and prospects. Renew Sust Energ Rev, 2013; 19: 360-369.
4. Capri E, Civita M, Corniello A, Cusimano G, Maio MD, Ducci D et al. Assessment of nitrate contamination risk : The Italian experience. J Geochem Explor, 2009; 102: 71-86.
5. Chini Zitelli G, Pastorelli R, Tredici MR. A modular Flat Panel Photobioreactor (MFPP) for indoor mass cultivation of *Nannochloropsis* sp. under artificial illumination. J Appl Phycol, 2000; 12: 521-526.

6. Cho S, Lee N, Park S, Yu J, Thao LT, Oh YK. Microalgae cultivation for bioenergy production using wastewaters from a municipal WWTP as nutritional sources. Biores Technol, 2013; 131:515-520
7. Di Maria F, Sordi A, Cirulli G, Gigliotti G, Massaccesi L, Cucina M. Co-treatment of fruit and vegetable waste in sludge digesters. An analysis of the relationship among bio-methane generation, process stability and digestate phytotoxicity. Waste Manag, 2014; 34: 1603-1608.
8. Franchino M, Comino E, Bona F, Riggio VA. Growth of three microalgae strains and nutrient removal from an agro-zootechnical digestate. Chemosphere, 2013; 92:738-744.
9. Gell K, Groenigen JV, Luz M. Residues of bioenergy production chains as soil amendments: immediate and temporal phytotoxicity. J Hazar Mater, 2011; 186: 2017-2025.
10. ISPRA. Procedura di misurazione per la determinazione della Richiesta Chimica di Ossigeno (COD) mediante test in cuvetta: Metodo 5135. Manuali e line guida ISPRA 117/2014. Roma: ISPRA; 2014.
11. Li X, Guo J, Dong R, Ahring BK, Zhang W. Properties of plant nutrient : Comparison of two nutrient recovery techniques using liquid fraction of digestate from anaerobic digester treating pig manure. Sci Total Environ, 2016; 544: 774-781.
12. Kumar MS, Miao ZH, Wyatt SK. Influence of nutrient loads, feeding frequency and inoculum source on growth of *Chlorella vulgaris* in digested piggery effluent culture medium. Bioresour Technol, 2010; 101:6012-6018.
13. Nkoa R. Agricultural benefits and environmental risks of soil fertilization with anaerobic digestates: a review. Agron Sustainable Dev, 2014; 34:473-492.
14. Park J, Jin HF, Lim BR, Park KY, Lee K. Ammonia removal from anaerobic digestion of livestock waste using green alga *Scenedesmus* sp. Bioresour Technol, 2010; 101: 8649-8657.

15. Piccinini S, Vismara R. Il biogas nel piano d'azione nazionale per le energie rinnovabili e il parco impianti in Europa e in Italia.In: Vismara R, Canziani R, Malpei F, Piccinini S. Biogas da agrozootecnia e industria. Dario Flaccovio Editore; 2011. p. 1-17.
16. R Development Core Team. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing. 2012 ISBN3-900051-07-0, Available at: http://www.R-project.org [accessed 30.09.15].
17. Singh M, Reynolds DL, Das KC. Microalgal system for treatment of effluent from poultry litter anaerobic digestion. Biores Technol, 2011; 102: 10841-10848
18. Šoštaric M, Golob J, Bricelj M, Klinar D, Pivec A. Studies on the growth of *Chlorella vulgaris* in culture media with different carbon sources. Chem Biochem Eng, 2009; 23: 471-477.
19. Tigini V, Franchino M, Bona F, Varese GC. Is digestate safe? A study on ecotoxicity and environmental risk of a piggery manure. Sci Tot Environ, 2016 in press.
20. Uggetti E, Sialve B, Trably E, Steyer JP. Integrating microalgae production with anaerobic digestion: a biorefinery approach. Biofuels Bioprod Bior, 2014; 8:516–529.
21. Xia A, Murphy JD. Microalgal cultivation in treating liquid digestate from biogas systems. Trends Biotech, 2016 in press.
22. Wang L, Li Y, Chen P, Min M, Chen Y, Zhu J, Ruan RR. Anaerobic digested dairy manure as a nutrient supplement for cultivation of oil-rich green microalgae *Chlorella* sp. Bioresour Technol, 2010; 101:2623-2628.

Annex I

Estimated coefficient (β), standard error and p-value for the predictor in the model relating the biomass growth (dry weight) of *C.vulgaris* to the different dilution rates (10% and 5%) at

different days (predictor: time) . For the categorical variable "dilution rate", we used the control treatment or the 10% dilution as reference levels

Reference level	Predictors	β	Standard Error	p-value
control	10%	-1.5487	0.1673	<0.001
	5%	-1.0356	0.1369	<0.001
	time	0.1570	0.0163	<0.001
10% dilution	5%	0.5132	0.1921	<0.01
	Control	1.5487	0.1673	<0.001
	time	0.1570	0.0163	<0.001